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Authors

Skowronska-Krawczyk, Dorota
Ma, Qi
Schwartz, Michal
[et al.](#)

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Required Enhancer: Matrin-3 Network Interactions for Pit1 Homeodomain Transcription Programs

Dorota Skowronska-Krawczyk^{1,*}, Qi Ma¹, Michal Schwartz^{1,2}, Kathleen Scully¹, Wenbo Li¹, Zhijie Liu¹, Havilah Taylor¹, Jessica Tollkuhn¹, Kenneth A. Ohgi¹, Dimple Notani¹, Yoshinori Kohwi³, Terumi Kohwi-Shigematsu³, and Michael G. Rosenfeld^{1,*}

¹Howard Hughes Medical Institute, Department of Medicine, School of Medicine, University of California, San Diego, La Jolla, California 92093, USA

²The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Israel

³Lawrence Berkeley National Laboratory, Berkeley, CA 94720

Abstract

Homeodomain proteins, described 30 years ago^{1,2}, exert essential roles in development as regulators of target gene expression^{3,4}, however the molecular mechanism underlying transcriptional activity of homeodomain factors remains poorly understood. Here, investigation of a developmentally-required POU-homeodomain transcription factor, Pit1/Pou1f1, has revealed that, unexpectedly, binding of Pit1-occupied enhancers⁵ to a nuclear matrin-3-rich network/architecture^{6,7} is a key event in effective activation of the Pit1-regulated enhancer/coding gene transcriptional program. Pit1 association with Satb1⁸ and β -catenin is required for this tethering event. A naturally-occurring, dominant negative, point mutation in human Pit1 (R271W), causing combined pituitary hormone deficiency (CPDH)⁹, results in loss of Pit1 association with β -catenin and Satb1 and therefore the matrin-3-rich network, blocking Pit1-dependent enhancer/coding target gene activation. This defective activation can be rescued by artificial tethering of the mutant R271W Pit1 protein to the matrin-3 network, bypassing the prerequisite association with β -catenin and Satb1 otherwise required. The matrin-3 network-tethered R271W Pit1 mutant, but not the untethered protein, restores Pit1-dependent activation of the enhancers and recruitment of co-activators, exemplified by p300, causing both eRNA transcription and target gene activation. These studies have thus revealed an unanticipated homeodomain factor/ β -catenin/Satb1-dependent localization of target gene regulatory enhancer regions to a subnuclear architectural structure that serves as an underlying mechanism by which an enhancer-bound homeodomain factor effectively activates developmental gene transcriptional programs.

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*Corresponding authors: dkrawczyk@ucsd.edu, mrosenfeld@ucsd.edu.

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Author Contributions: D.S-K and M.G.R. conceived the project. D.S-K performed the majority of the experiments; Q.M. performed the bioinformatic analyses; M.S. contributed 3D-ImmunoFISH. W.L. contributed GRO-Seqs; K.O. assisted in deep-sequencing library preparations and sequencing; Additional experiments/methods were contributed by K.S., J.T., Z.L. and D.N.; H.T. and K.S. assisted in animal based experiments. Y.K. and T.K-S. contributed critical insights and reagents. D.S-K., K.S. and M.G.R. wrote the manuscript. All authors discussed the results and commented on or edited the manuscript.

During pituitary development, the POU-homeodomain transcription factor, Pit1, is necessary for differentiation of thyrotrope, lactotrope, and somatotrope cell types in both mice and humans^{10,11}. To further understand the molecular basis for Pit1-mediated gene activation, we mapped the genomic localization of Pit1 by ChIP-seq using specific Pit-1 antibody (Fig. S1a, b) in a growth hormone (GH)-expressing rat pituitary cell line (GC). Of 14,466 Pit1 binding sites identified, >80% overlapped with H3K4me² histone marks but not with transcription start sites, indicative of enhancer elements⁵ (Fig. 1a, b, Supplementary Table 1).

To identify Pit1 interacting factors, we immunoprecipitated HA-tagged Pit1 from 293T cells, and using mass spectrometry, identified matrin-3 and hnRNPU as two highly abundant proteins interacting with Pit1 (Fig. 1c, Supplementary Table 2, 3). The interactions were confirmed by co-immunoprecipitation (co-IP)/Western blot experiments in 293T cells (Fig. S1c) and with endogenous proteins in GC cells (Fig. 1d, Fig. S1d). Both matrin3 and hnRNPU have been previously found in a salt extraction-resistant nuclear fraction, as part of an internal fibrogranular network^{6,12,13} which we term the nuclear matrin-3-rich network (Fig. S1e). These proteins bind DNA at sites known as matrix/scaffold attachment regions¹³. ChIP-Seq using a specific α -matrin-3 antibody revealed that >50% of matrin-3 binding sites co-localized with H3K4me² peaks (Fig. S1f), showing significant enrichment (~16 fold, p-value<1e-100) over the ~3% predicted random co-localization with H3K4me² peaks, covering ~5% of the rat genome. Of these matrin3/H3K4me² sites, ~80% were elements distal to transcription start sites (Fig. 1e). This finding provides an initial demonstration of matrin3 association with DNA regulatory sequences in the genome. We noticed that Pit1-associated H3K4me², H3K4me¹ and H3K27Ac-marked enhancers, co-localized with matrin-3 (Fig. 1f–i, Fig. S1g), and confirmed matrin-3 antibody specificity by knockdown of endogenous matrin-3 protein (Fig. S1h, i). To investigate whether regulatory elements bound by Pit1 occupy the same nuclear compartment as matrin-3, we performed immunofluorescence (FISH) experiments with DNA-FISH probes specific to the growth hormone (*GH*) locus and α -matrin-3 antibodies, finding that >65% of the FISH signals co-localized with matrin-3 (Fig. 1j, Fig S1j).

Because the pituitary specific homeodomain protein, Prop1, interacts with β -catenin to activate target gene expression¹⁴, we investigated β -catenin interacting partners in differentiated pituitary cells by expressing β -catenin fused to the biotin ligase recognition peptide (BLRP), along with biotinylating enzyme BirA, in GC cells. Biotinylated β -catenin was present in both the cytoplasmic and the nuclear compartments (Fig. 2a, Fig. S2a). Streptavidin pulldown, followed by mass spectrometry, revealed that while cytoplasmic β -catenin interacting factors included α -catenin, as previously described¹⁵, Pit1 peptides predominated in the nuclear β -catenin fraction (Fig. 2a, Supplementary Table 4–7). This putative Pit1: β -catenin interaction was confirmed between endogenous proteins by co-IP/Western blot experiments using nuclear extracts from GC pituitary cells (Fig. 2b). Using GST-pulldown assays, armadillo repeat 8 of β -catenin proved sufficient to mediate this interaction, exactly within a region that has been previously described to interact with Prop1¹⁴ as well as Lef/Tcf¹⁶ (Fig 2c, Fig S2b).

We then examined whether β -catenin plays a role in regulating expression of Pit1 target genes, such as GH. Effective knockdown of β -catenin in GC cells (Fig. S2c) dramatically decreased the level of nascent GH transcripts (Fig. 2d), analogous to what was observed when *Pit1* was knocked down (Fig. 2d, Fig. S2c). β -catenin also forms a complex with Satb1¹⁷, a well-established genome organizer essential for multiple biological processes, including T-cell activation and cancer progression^{8,18,19}. RT-qPCR analysis revealed that the upregulation of Satb1 expression in the mouse pituitary gland coincides with differentiation of Pit1 lineages during pituitary development (Fig. S2d), and immunohistochemical analysis confirmed that SATB1 protein is expressed in somatotropes and other cells of the mouse pituitary gland (Fig. S2e). Co-IP of Pit1 from GC cells revealed an interaction with Satb1 (Fig. 2b) which was reproduced by co-IP of HA-tagged Pit1 in 293T cells (Fig. S2f). Indeed, conditional knock-out of *Satb1* in the mouse anterior pituitary significantly decreased expression of *GH* (Fig. S2g). We therefore investigated whether interaction between Pit1 and Satb1 was dependent on β -catenin, and vice-versa. Exogenously expressed, HA-tagged Pit1 protein was pulled down after knocking down either β -catenin or *Satb1* in 293T cells. In the absence of β -catenin, the interaction between Pit1 with Satb1 was diminished, indicating a role for β -catenin in mediating Pit1:Satb1 interaction. Reciprocally, the interaction of Pit1 with β -catenin was partially abolished by *Satb1* knockdown (Fig 2e).

Pit1 has been shown previously to co-fractionate with the insoluble, matrin-3 rich, nuclear fraction in *in-vitro* biochemical studies²⁰. We therefore used lithium 3,5-diiodosalicylate (LIS), a chaotropic reagent that extracts a majority of nuclear proteins but preserves the nuclear matrin-3-rich network²¹, to investigate Pit1 subnuclear locations (Fig. S2h). We observed that, in somatotropes, Pit1 indeed co-fractionated with matrin3 and Satb1 in the LIS resistant insoluble fraction, while β -catenin was partitioned between the insoluble and soluble fractions (Fig. S2i). When both β -catenin and Satb1 proteins were simultaneously depleted (Fig. S2j), the presence of Pit1 in the LIS resistant insoluble fraction was significantly reduced (Fig. 2g). In the double knockdown, total Pit1 protein levels were unchanged and Pit1 protein remained localized to the nucleus (Fig. S2k); however, it was now detectable in the looped-out DNA fraction obtained after DNAase I digestion of nuclear halos (Fig. S2l). Together, these data indicate that localization of Pit1 protein to the insoluble fraction is dependent on interaction with β -catenin and Satb1. When endogenous Pit1 protein was pulled down before and after knocking down both β -catenin and Satb1 in GC cells, the absence of these proteins highly affected interaction between Pit1 and matrin-3 (Fig. 2g).

To further investigate whether association of Pit1-bound enhancers with the matrin-3 fraction is indeed dependent on β -catenin and SATB1, we performed immuno-FISH experiments in GC cells with α -matrin-3 antibodies and DNA-FISH probes specific to the growth hormone (*GH*) locus. In GH-expressing GC cells, ~65% of the FISH signals co-localized with matrin-3. When both β -catenin and Satb1 were depleted, only ~35% of FISH signals loci co-localized with matrin-3, similar to the overlap of a GH locus in a Pit-1 positive, GH-non-expressing cell line (MMQ) (Fig. 2h, **S2m**). We thus conclude that association of Pit1 enhancers with the nuclear matrin-3-rich network/structure observed by

immuno-FISH (Fig. 1j, Fig. 2h) and biochemical extractions (Fig. 2f Fig. S2h,) is dependent on intact interactions of Pit1 with both β -catenin and Satb1.

Global run-on (GRO-seq) analysis using GC cells before and after simultaneous knockdown of both β -catenin and Satb1 revealed that in total, 1350 coding gene nascent transcripts were downregulated and 916 genes were upregulated under these double-knockdown conditions (Fig. S3a). Out of the 1350 genes positively regulated by both β -catenin and SATB1 (Supplementary Table 8), 991 had Pit1 bound enhancer elements within 200kbp of their start sites (Fig. S3a), and all 991 were significantly downregulated (Fig. 3a). Consistent with their functional importance²² the expression of the eRNAs associated with these 991 Pit1-bound enhancers was significantly affected in the simultaneous knockdown of *β -catenin* and *Satb1* (Fig. 3b). ChIP-seq analysis revealed that Satb1 and β -catenin proteins were located at the center of Pit1-positive enhancers (Fig. S3b–d), strongly supporting the co-binding model of these three proteins. Based on this analysis, we selected several highly down-regulated genes that contain Pit1 enhancers (Fig. S3c) and observed that knockdown of either of the two proteins significantly diminished target gene expression (Fig. S3g). Additionally, knockdown of either *Pit1* or *β -catenin* affected Satb1 association with enhancer elements, as assessed by ChIP-qPCR (Fig. 3c, Fig. S3e). Association of matrin-3 with Pit-1 dependent enhancers was significantly reduced when either β -catenin or SATB1 were downregulated (Fig. S3h). Finally, we confirmed that lack of Pit1 protein inhibited association of selected Pit1-dependent enhancers with matrin-3 (Fig. 3d, Fig. S3f). Together, these data suggest that functional interaction between Pit1-dependent regulatory elements and matrin-3 requires association of the DNA-bound Pit1 with β -catenin and SATB1. Consistent with this suggestion, lack of matrin3 had a negative effect on the expression of target genes (Fig. S3i).

Naturally occurring mutations in Pit1 underlie number of combined pituitary hormone deficiency (CPHD) cases in humans, with the R271 residue being the most frequently mutated⁹. An amino acid 271 maps to the last turn of helix3 in the homeodomain and is located outside the DNA binding surface. While the R271W mutation in Pit1 confers dominant-negative properties (Fig. S4a)⁹, it does not affect protein stability or binding to its cognate DNA sites (Fig. S4b–d)⁹. We therefore investigated whether the R271W mutation affects the composition of the Pit1 complex. Co-IP of recombinant HA-tagged Pit1 proteins followed by Western blot analysis revealed that the R271W mutation disrupted the ability of Pit1 to interact with matrin3 and hnRNPU, apparently due to its inability to bind β -catenin and Satb1 (Fig. 4a). Additionally, biochemical fractionation indicated that the R271W mutant is largely lost from the insoluble fraction (Fig. 4b), although both, WT and R271W proteins were expressed at similar levels and found entirely localized to the nucleus (Fig. S4e). Instead R271W was detected in the ‘looped-out DNA’ fraction (Fig. 4b), suggesting that it had lost its ability to interact with the LIS resistant fraction.

Together, these data suggested that a key function of homeodomain transcription factors such as Pit1 might be to join bound regulatory regions to the matrin-3-rich network in an association required to activate target gene transcription. Because the transcriptionally-inactive R271W mutation interferes with this association, we experimentally tested this hypothesis by artificially tethering the R271W mutant to the matrin-3 network to try to restore target gene transcription. Several protein domains that are responsible for association

with the matrin-3-rich “matrix attachment regions” have been previously described and include the SAF/SAP domain (e.g. in hnRNPU²³, SAF-B, PARP proteins²⁴); AT-hooks (HMG proteins²⁵); and the atypical homeodomain, SATB1¹⁸. We therefore grafted the SAF/SAP domain from rat hnRNPU (AA 4–45), onto HA-tagged wild type and R271W Pit1 (Fig. 4c). As predicted, R271W-SAF hybrid protein localized efficiently in the insoluble nuclear fraction (Fig. S4f). Hybrid WT-SAF and R271W-SAF proteins were expressed in GC cells treated with siRNAs to deplete endogenous Pit1 (Fig. S4g). As expected, matrin3 association with Pit1-dependent enhancers and expression of Pit1-dependent coding genes and eRNAs were lost upon *Pit1* knockdown but were restored in the presence of either WT or WT-SAF Pit1 (Fig. 4d, Fig. S4i). In contrast, neither the association of Pit1 enhancers with matrin-3 nor target gene and eRNA expression were rescued in R271W-expressing cells. However, the R271W-SAF hybrid rescued matrin-3 interaction and Pit1 transcriptional activation to levels close to that achieved by wild-type Pit1. These experiments indicate that specific subnuclear localization of Pit1 is a *bona fide* requirement for its full activity, and that this is the only functional defect in the R271W mutant Pit1 protein.

To investigate which step of enhancer activation is affected by R271W, we tested recruitment of CBP/p300, a known co-factor of Pit1²⁶, finding that p300 association with tested enhancer elements was significantly reduced (Fig. S4h). Overexpression of R271W was not able to rescue this recruitment while hybrid R271W-SAF did restore p300 association with Pit1 bound enhancers (Fig. S4h). These data suggest that eRNA transcription and binding of critical co-activators, such as p300/CBP, require recruitment of Pit1 to the matrin-3-rich network.

To further substantiate these observations in intact pituitary, *ex vivo* rescue approach took advantage of the “Snell” mouse model that harbors a mutation (W261C) in Pit1 that renders it incapable of binding to cognate DNA sites. Overexpressing HA-tagged Pit1-R271W-SAF hybrid, but not Pit1-R271W protein, rescued expression of endogenous GH as visualized by immunohistochemistry (Fig. 4e). Based on these data, we propose a model in which Pit1 association with the matrin-3-rich network is indispensable for its biological activity (Fig. 4f), as demonstrated by the lack of transcriptional activity of the naturally occurring mutant R271W Pit1 (Fig. 4f).

Nuclear organization and genome 3D structure are postulated to play a prominent role in regulation of gene expression^{28,29}. Here, we have presented evidence that a homeodomain transcription factor regulates gene transcriptional programs through interaction with components of subnuclear structure (e.g. matrin-3 rich structure). Other homeodomain proteins (e.g. Lhx3, Prop1, Oct1), as well as different classes of transcription factors³⁰, have been shown to associate with the high salt-extraction resistant fraction, corresponding to the matrin3-rich network. Therefore, our data suggest a model in which this interaction may be required for transcriptional activation for many homeodomain proteins, helping to solve a long-standing gap in our understanding of gene activation programs by this important class of DNA binding transcription factors, and perhaps several other types of DNA binding transcription factors, revealing that the regulated enhancer activation requires localization in a specific subnuclear architectural structure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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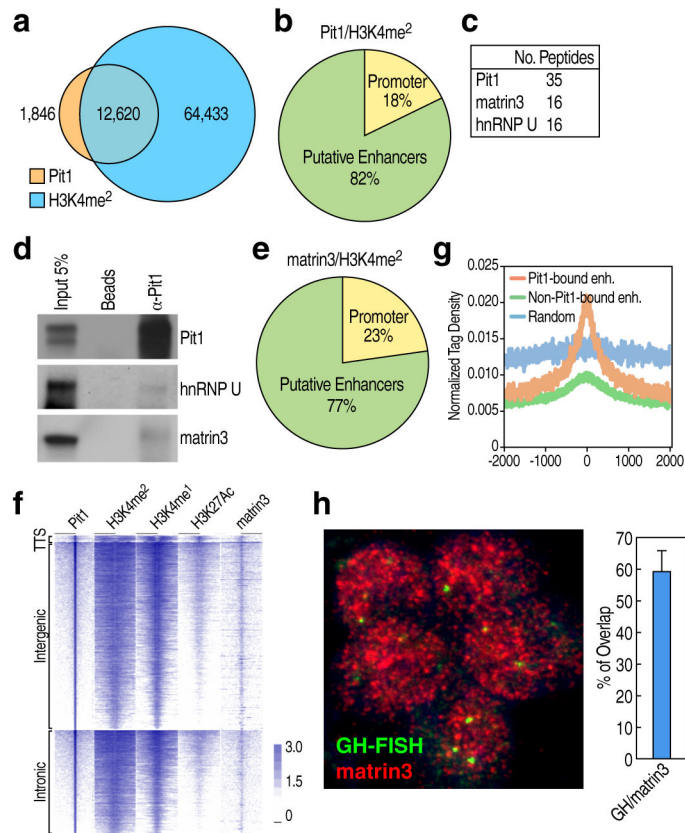


Figure 1. Co-localization of Pit1 and matrin-3 on enhancers

a, ChIP-Seq analysis of Pit1 and H3K4me² genome-wide co-localization in GC rat pituitary somatotrope cell line. **b**, Most DNA sites co-bound by Pit1 and H3K4me² lie outside gene proximal promoters. **c**, HA-tagged Pit1 immunoprecipitated from 293-T cells. Co-purifying factors identified with mass spectrometry. **d**, Co-immunoprecipitation of Pit1 followed by Western blot analysis confirmed Pit1: endogenous matrin 3 and hnRNP U interactions in GC cells. **e**, Most matrin-3/H3K4me² sites in GC cells lie outside gene proximal promoters. **f**, Heat map of ChIP-Seq data on non-promoter genome-wide association of Pit1, H3K4me², H3K4me¹, H3K27Ac, matrin-3 in GC cells centered on Pit1 sites and categorized as transcription termination sites (TTS), intergenic, intronic sites. **g**, Meta-analysis of matrin-3 ChIP-seq data **h**, Examples of immuno-FISH experiments showing matrin3 spots (red) colocalized with GH locus spots (green) in GH-expressing GC cells. Chart represents count of percentage of signals exhibiting co-localization, n 200, +/- SD.

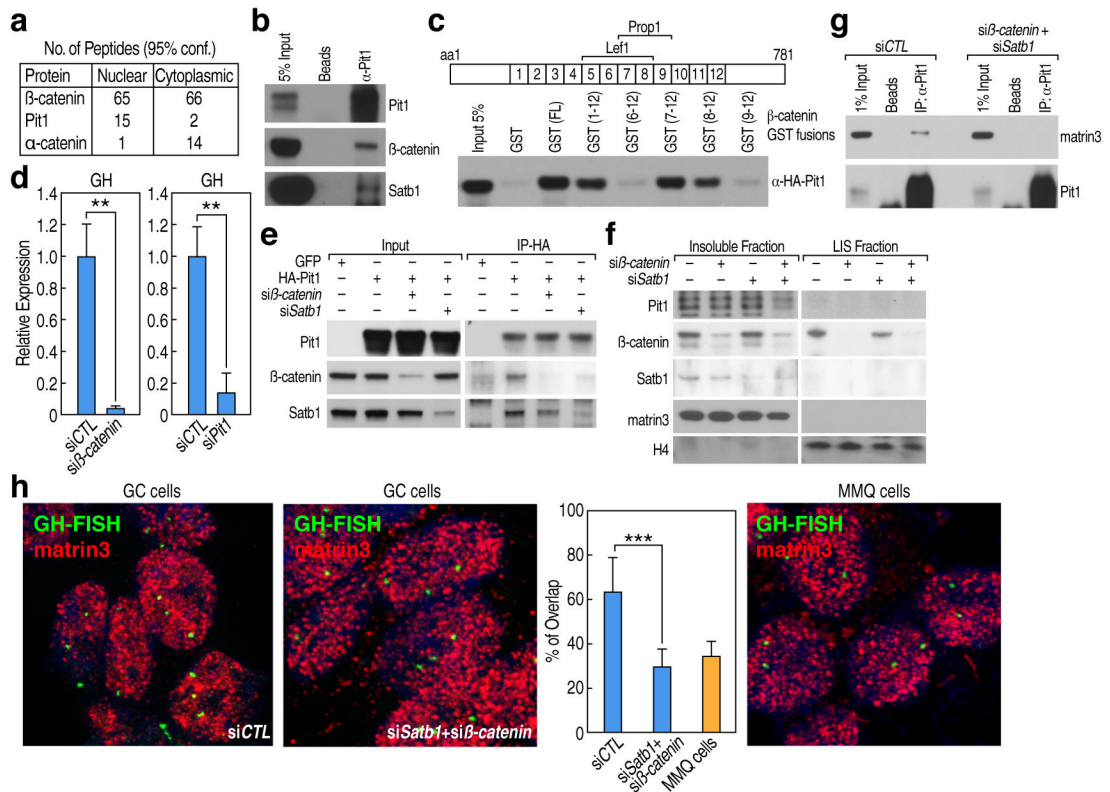


Figure 2. Pit1 association with LIS resistant nuclear component is β -catenin- and SATB1-dependent

a, BLRP- β -catenin immunoprecipitated from cytoplasmic or nuclear fractions of GC cells. Co-purifying factors identified by mass spectrometry. **b**, Co-IP-Western analysis confirmed Pit1: β -catenin interaction and revealed interaction with SATB1. **c**, GST-pulldown showing β -catenin armadillo repeat 8 (within region previously shown to interact with Prop1 and Lef1) is required for interaction with Pit1. **d**, Nascent GH transcripts levels after siRNA knockdown of β -catenin and Pit1 analyzed by RT-qPCR. Experiments were repeated 2 times, and p-values calculated using student's two tailed t-test. (+/- SD; **p<0.01) **e**, Co-IP of HA-tagged Pit1 protein in 293T cells before and after β -catenin and Satb1 knockdown showing Pit1 interacts simultaneously with both proteins. **f**, LIS nuclear extraction before and after β -catenin or/and Satb1 knockdown shows that both proteins are needed for Pit1 retention in LIS resistant fraction. **g**, Co-IP of Pit1 protein in GC cells before and after simultaneous knockdown of β -catenin and Satb1 shows interaction of Pit1 with matrin-3 is dependent on both. **h**, Examples of immuno-FISH experiments showing GH locus (green) colocalizing with matrin3 (red) with higher frequency in control culture conditions than after siRNA β -catenin and siRNA Satb1 treatment in GC cells. In Pit1 positive, non-GH-expressing MMQ cells, significantly fewer GH loci are associated with matrin3. The chart represents the count of percentage of signals exhibiting co-localization in control vs. si β -catenin and siSATB1 conditions compared to MMQ cells; n 200, +/- SD, ***p<0.001

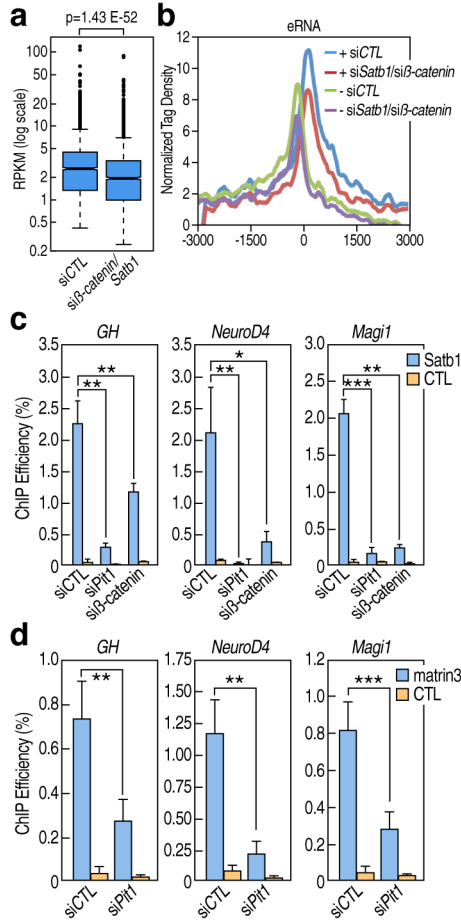


Figure 3. β -catenin and SATB1 influence transcription of genes regulated by Pit1 enhancers
a, Genes containing Pit1 enhancers (991) are significantly downregulated upon knockdown of β -catenin and *Satb1*. RPKM – reads per kilobase per million **b**, GRO-seq tag distribution on both strands of Pit1 bound enhancers before and after treatment of GC cells with β -catenin and *Satb1* siRNAs. **c**, ChIP-qPCR analysis of *Satb1* association with *Pit1* enhancers upon either *Pit1* or β -catenin knockdown. Experiments were repeated 3 times, and p-values calculated using student’s two tailed t-test. (+/- SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) **d**, ChIP-qPCR analysis of matrin-3 association with Pit1 enhancers upon *Pit1* knockdown. Experiments repeated 2–4 times, p-values calculated using student’s two tailed t-test. (+/- SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

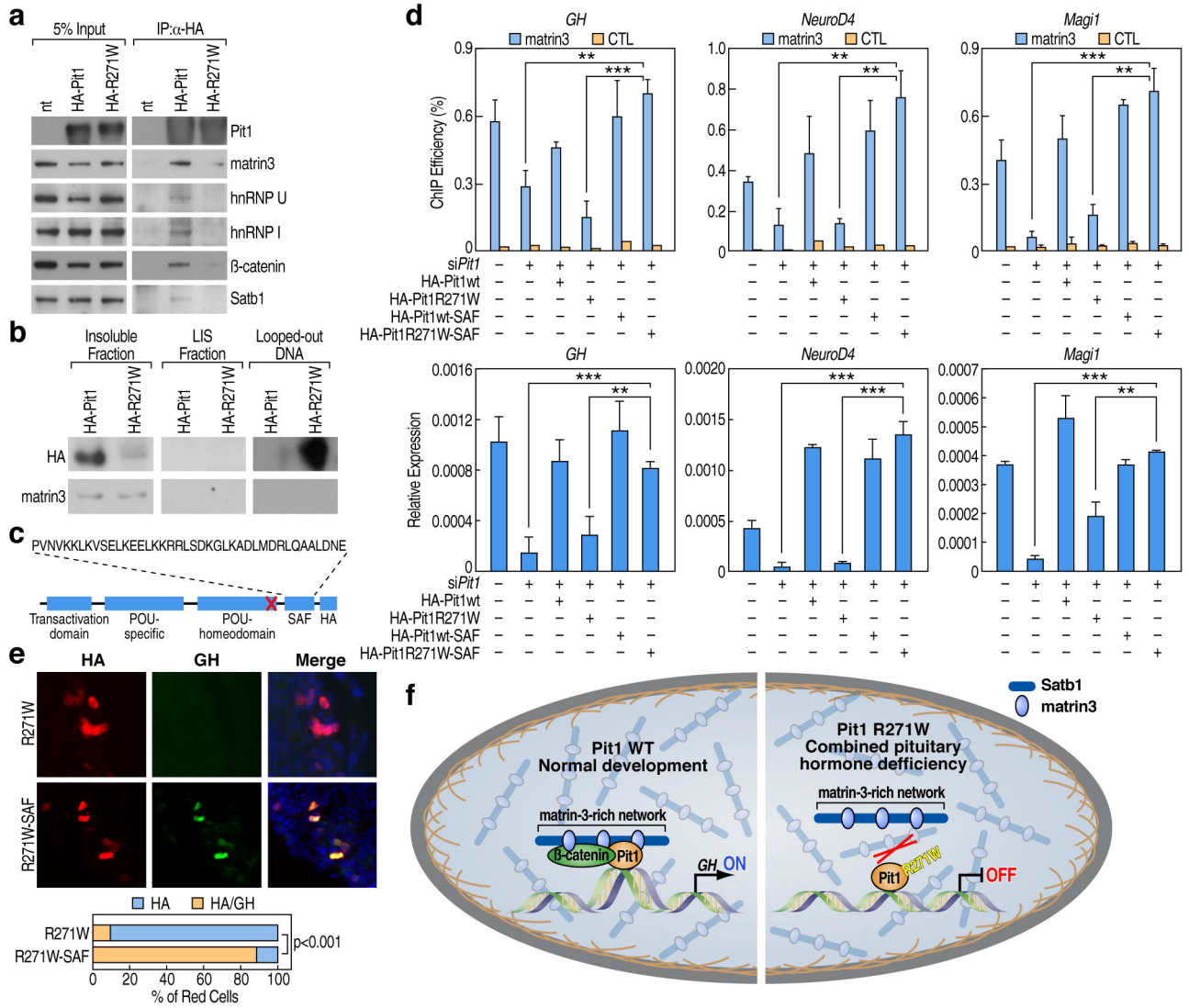


Figure 4. Naturally occurring R271W mutation in human Pit1 affects ability of Pit1 to interact with matrin-3 rich network

a, Western blot analysis showing defective interaction of Pit1R271W mutant with components of nuclear matrin-3 enriched network as well as β-catenin and Satb1. **b**, Biochemical extraction shows that the amount of R271W mutant protein decreased in the LIS-resistant fraction and associates instead with the looped-out DNA fraction. **c**, Schematic representation of Pit1 protein with added SAF domain. red “x” – location of amino-acid 271 **d**, Analysis of effect of overexpression of different forms of Pit1 protein in absence of endogenous Pit1; *top panels*: ChIP-qPCR analysis of matrin-3 association; *bottom panels*: RT-qPCR analysis of transcriptional effect on selected targets. Experiments were repeated 3 or 4 times, and p-values calculated using student’s two tailed t-test. (+/- SD; **p<0.01, ***p<0.001) **e**, Analysis of effect of *ex vivo* overexpression of R271W mutant with and without SAF domain in e17.5 *snell* mutant pituitary glands. GH expression is rescued only when the R271W-SAF protein is overexpressed. *Bottom*: quantification of percentage of HA-positive GH expressing cells. Two pituitaries were used per condition; p-value

calculated using χ^2 test. **f**, Model: Association of Pit1-bound regulatory element with matrin-3 rich network and loss of this interaction based on failure of R271W mutant to interact with β -catenin and SATB1 causing loss of gene activation as in CPHD.

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